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Using Gordiid cysts to discover the hidden diversity, potential distribution, and new species of Gordiids (Phylum Nematomorpha)

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Abstract

In this study, we sampled aquatic snails for the presence of hairworm cysts from 46 streams in Payne County, Oklahoma. Gordiid cysts were found at 70 % (32/46) of sites examined. Based on cyst morphology, we were able to identify three morphological types of gordiid cysts, including *Paragordius*, *Gordius*, and *Chordodes*/*Neochordodes*. Using our gordiid cyst presence data in conjunction with environmental variables, we developed an ecological niche model using Maxent to identify areas suitable for snail infections with gordiids. The model successfully predicted all presence localities of gordiid cysts in snails over a geographic area of 1,810 km^2. We used this information, along with arthropod host infections and crowdsourcing, citizen scientists sampling for adult free-living worms during peak emergent times in areas predicted suitable by the model, to document *Paragordius varius*, *Chordodes morgani*, and a new species of gordiid (*Gordius* **n. sp.**). To our knowledge, this is the first ecological niche model attempted on such a narrow geographic scale (county level) that recovered known locations successfully. We provide new scanning electron micrographs and molecular data for these species. Our field data and ecological niche model clearly indicate that gordiid cysts are easy to detect in the environment and together these sampling techniques can be useful in discovering new species of gordiids, even in relatively well sampled areas for these cryptic parasites.

Key words: Gordiida, hairworm, Gordian worm, North America, scanning electron microscopy, differential interference contrast microscopy, non-adult life stages, ecological niche models, *Chordodes*, *Gordius*, *Paragordius*, *Neochordodes,* COI sequences

Introduction

Freshwater nematomorphs, commonly known as hairworms or gordiids, have been identified as one of the most understudied groups of parasites (Poulin 1998). One reason for the poor knowledge of the diversity and distribution of hairworms is the lack of reliable ways to collect the free-living adults over large geographic areas. However, recent work indicates that cyst stages may be the most commonly encountered gordiid life stage in the environment (Hanelt *et al*. 2001; Hanelt *et al.* 2012; Bolek *et al.* 2013a). Cysts of gordiids have been reported to be long lived in their hosts and are found in a variety of aquatic invertebrate and vertebrate paratenic hosts (Hanelt & Janovy 2003). However, Hanelt *et al.* (2001) argued that aquatic snails are the most suitable indicator hosts to sample for gordiid cysts over wide geographic areas. Three major reasons for this argument include the wide and common distribution of snails in aquatic environments, the lack of immunological response of snails to gordiid cysts, and the ease of processing snails for gordiid cysts compared to other aquatic invertebrates (Hanelt *et al*. 2001; Bolek & Coggins

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2002; Bolek *et al.* 2010; Hanelt *et al.* 2012; Bolek *et al.* 2013a). This is particularly important because an ideal indicator host should maintain a parasitic infection for long periods of time, allowing investigators to track the occurrence of that parasite long after other stages of that parasite have disappeared from a particular geographic location. Additionally, the feeding behavior of snails on the bottom of aquatic habitats makes them ideal hosts to encounter gordiid larvae, which reside in these microhabitats (Hanelt *et al.* 2001; Bolek *et al.* 2015). As a result, snails are likely to come in contact with the microscopic and semi-sessile gordiid larvae more commonly than other invertebrates and vertebrates in aquatic habitats (Hanelt *et al.* 2005).

While traditionally adults are needed for species identification, our work on non-adult stages of gordiids indicated that the folding pattern of the encysted larvae can be used in generic and/or clade identification of gordiid cysts (Szmygiel *et al.* 2014). Using this information, Hanelt *et al.* (2012) and Bolek *et al.* (2013a) collected snails infected with two types of gordiid cysts from Kenya, a country for which no gordiid records existed. Exposure of the cysts to the appropriate group of laboratory-reared arthropods yielded adult gordiids which could be identified and led to the discovery of two new species (Hanelt *et al.* 2012; Bolek *et al.* 2013a). Taken together, these recent advances on describing the morphology of gordiid cysts and our understanding of hairworm life cycles have allowed the use of cyst stages to investigate the biodiversity and distribution of these cryptic parasites at a much larger geographic scale than with adult stages alone.

Although snail field surveys can be useful in identifying the local geographic distribution of gordiids, additional tools are needed to predict the broader range of gordiids in order to conduct further studies on the distribution of these parasites. Ecological niche modeling is one such method which has received recent attention as a powerful tool for characterizing ecological and geographic distributions of cryptic species across landscapes (Peterson 2003; Raxworthy *et al.* 2003). This modeling technique has enabled researchers to predict the potential distributions of parasites even though little information is available on a particular parasite species life-cycle or its biology (Peterson 2006; Peterson *et al.* 2002). This approach has allowed researchers to generate potential species distribution maps that can provide additional information in forming accurate predictions on potential intermediate, paratenic, definitive or reservoir hosts for specific species of parasites where such information is lacking (Haverkost *et al.* 2010; Peterson *et al.* 2002). This method is especially appealing for studying gordiids because virtually nothing is known about the distribution, life cycles or host use for most hairworm species (Schmidt-Rheasa *et al.* 2003; Bolek *et al.* 2015). While recent progress has been made in understanding the general life-cycle of hairworms, many questions regarding their paratenic and definitive host use in nature remain unanswered (Schmidt-Rhaesa 1997; Hanelt & Janovy 1999; Hanelt & Janovy 2003; Hanelt & Janovey 2004; Schmidt-Rhaesa *et al.* 2003; Hanelt *et al.* 2005; Schmidt-Rhaesa *et al.* 2008; Bolek *et al.* 2010; Hanelt *et al.* 2012; Schmidt-Rhaesa 2013; Bolek *et al.* 2013a; Bolek *et al.* 2013b; Bolek *et al.* 2015).

To evaluate the use of these novel methodologies for biodiversity studies of gordiids, we sampled snails for gordiid cysts in Payne County, Oklahoma, U.S.A. We had three objectives in this study. First, by using the cyst stage of gordiids infecting snail hosts, we aimed to document the distribution of gordiids in Payne County, Oklahoma. Second, we tested if cyst morphology could be useful for expanding our knowledge of the biodiversity of gordiids in Oklahoma because at the time of this study only a single species of gordiid (*Gordius robustus* Leidy, 1851) was known from Oklahoma (Hanelt & Janovey 2002; Schmidt- Rhaesa *et al*. 2003; McAllister *et al.* 2013). Finally, using our field presence data of gordiid cysts, we made predictions about gordiid distribution by developing ecological niche models. To meet these objectives, we sampled for freshwater snails in small (first and second order) streams from Payne County, Oklahoma. Cyst morphology and either arthropod host infections or collections of adult free-living worms were then used to identify the cysts to clade, genus, and/or species. Finally, gordiid cyst presence data and environmental variables were then used to develop an ecological niche model using Maxent to identify areas suitable for snail gordiid infections.

Methods

Snail collection, cysts processing, and cyst identification: Snail sampling was conducted during May through July of 2011. Forty-six first or second order streams were identified throughout Payne County, Oklahoma (1,825 km^2; Table 1), and approximately twenty individuals of *Physa acuta* Draparnaud, 1805 snails were collected by hand or dip-net from each stream. All sampling localities were geo-referenced using a hand-held Global

Positioning System (GPS) unit. Snails from each location were placed in labeled and capped 50 ml plastic centrifuged tubes, filled with approximately 35 ml of stream water, and brought back to the laboratory. Centrifuge tubes with snails were then frozen at -80 °C until examination. Before dissection, centrifuge tubes were thawed and all snails were removed. For each snail, the length and width of the shell was measured to the nearest 1.0 mm and each snail was processed for gordiid cysts following modifications of Hanelt *et al.* (2001). Briefly, the snail body was removed with forceps from its shell under a dissection microscope and then pressed between two slides. Once snail tissue was flattened, a wet mount was prepared by removing the top slide and adding a drop of water and covering the flattened tissue with a cover-slip. Slides were then examined with an Olympus BX-51 upright research microscope configured for bright field and differential interference contrast microscopy with plain fluorite objectives and a calibrated ocular micrometer at 100 x to 400 x total magnification. The status and degree of infection were determined by scanning the entire flattened snail carcass for cysts at 100 x to 400 x total magnification. The folding pattern for all cysts and length measurements of unencysted larvae within each infected snail at each site were recorded and digital photographs were taken for all cysts and larvae in snails with cyst/larva intensities of less than 20 with an Olympus 5 megapixel digital camera. Additionally, digital photographs were taken for at least 20 cysts/larvae per infected snail for snails with higher intensities than 20 cysts and/or larvae. The folding pattern of larvae within all fully formed cysts and the size of unencysted larvae in each snail were compared to morphological characteristics provided in Szmygiel *et al.* (2014). Briefly, cysts were grouped into three categories: folded once with small visible spines on the preseptum (species in the genera *Chordodes* Creplin, 1847 and *Neochordodes* Carvalho, 1942), folded twice with distinct large spines on the preseptum (species in the genus *Paragordius* Camerano, 1897), or folded twice without spines on the preseptum (species in the genus *Gordius* Linné, 1758). Unencysted larvae in snail tissue were identified to *Chordodes*/*Neochordodes* spp., *Paragordius* spp. or *Gordius* spp. based on length measurements of the preseptum and postseptum and the presence or absence of long outer hooks on the outer ring of the preseptum and then compared to measurements and morphological characteristics for larvae of gordiids reported in Szmygiel *et al.* (2014). Prevalence, the mean intensity (MI) and range, and mean abundance ± 1 standard deviation (MA ± 1 SD) were calculated for each location sampled according to Bush *et al*. (1997). Pearson's correlations were calculated for snail shell length and width and intensity or abundance of larvae/cysts for each site and the overall collection (Sokal & Rohlf 1981).

To confirm species identification of gordiids, adult worms were either reared from field collected cysts in commercially reared crickets, *Acheta domesticus* Linné, 1758 (a known host for *Paragordius* spp.), or free-living adult worms were collected by us or citizen scientists who first reported them to us though our website (www.nematomorpha.net) and its Report-A-Worm feature. All live worms were brought to the laboratory at Oklahoma State University and processed for morphological and/or molecular identification. For laboratory infections, crickets were exposed to defrosted *Paragordius* spp. cysts from field infected snails from four localities where *Paragordius* cysts occurred at high prevalence and intensities (sites 24, 27, 33, and 40; see Table 1), according to Bolek *et al.* (2013b). Infected snails with *Paragordius* cysts from these locations were frozen at -80 ºC for three months before being defrosted and used for cricket exposures. Exposed crickets were maintained in groups of up to 15 individuals in covered plastic shoe boxes (35 cm x 25 cm x15 cm) with a paper-towel-substrate and a 4 cm^2 egg carton for a hiding place. Crickets were watered and fed *ad libitum* by placing a 50 mm plastic centrifuge tube filled with aged tap-water with a cotton ball at the end, and given *Purina*® *Puppy Chow*® dog food. After 28 days, all surviving exposed crickets were placed in 110 × 35 mm Stender dishes partially filled with aged tap water and allowed to release worms. This process was repeated daily until all worms emerged from their cricket hosts or until the cricket died.

For species identification of laboratory reared and/or field collected adult free-living worms, the length of live male and/or female worms was measured in mm with a ruler without stretching the worms; worms were then placed in a Petri dish with aged tap water and the diameter was measured with a calibrated ocular micrometer using a Wild Heerbrugg M5 stereomicroscope at 500x magnification. The color of each worm was recorded and some individuals were fixed in 100% ethanol for molecular work; others were fixed in 70% ethanol for morphological studies. For scanning electron microscopy (SEM) studies, 5–10 mm sections of the anterior, midbody, and posterior regions of one to four male and/or female individual worms of each species were cut, critical point dried, mounted on aluminum stubs, coated with gold palladium, and examined with a FEI Quanta 600 field emission gun ESEM with Evex EDS and HKL EBSD. Additionally, the anterior, midbody, and posterior 5-10 mm sections of some specimens were examined with a Wild Heerbrugg M20 light microscope (LM) and photographed with a Nikon

Coolpix S4 digital camera with a Martin Microscope adapter. All terminology of areoles follows Schmidt-Rhaesa *et al.* (2003) and Hanelt *et al.* (2012). Species identification was based on standard keys in Schmidt-Rhaesa *et al.* (2016) and comparisons to published species description and re-descriptions in Schmidt-Rhaesa *et al.* (2003).

Material deposited: Adult male *Chordodes morgani* Montgomery, 1898, mounted on a SEM stub; adult male and female *Paragordius varius* Leidy, 1851 in vials of 70% ethanol, two adult males *Gordius* **n. sp.**, one mounted on a SEM stub and one in a vial of 70% ethanol, and one adult female *Gordius* **n. sp.** in a vial of 70% ethanol were deposited in the Museum of Southwestern Biology-Parasitology Division, accession numbers *C. morgani* male: MSB Para 24561; *P. varius* male and female: MSB Para 24563 and 24564; *Gordius* **n. sp.** males and female: MSB Para 24562, 19255, and 19256.

DNA extraction, amplification, and sequencing of adult free-living worms: Molecular work was conducted on two individuals including a *Paragordius* sp. and a *Chordodes* sp. From each worm, a 0.5 cm section was cut, dried at room temperature, and used for DNA extraction using the E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek, Norcross, Georgia), following the manufacturer's instructions. Extracted DNA was stored at -70 C. Partial CO1 was amplified using GoTaq Flexi DNA Polymerase (Promega Corp. Madison, Wisconsin). The primers used were the universal CO1 primers (Folmer *et al*. 1994) LCO1490: GGT CAA CAA ATC ATA AAG ATA TTG G and HCO2198: TAA ACT TCA GGG TGA CCA AAA AAT CA, using standard PCR protocols. PCR reactions were analyzed by agarose gel electrophoresis, with the use of 1.0% agarose gels, stained with 0.5% GelRed Nucleic Acid Gel Stain (Biotium, Hayward, California), and visualized on a UV transilluminator. Amplicons were purified by ethanol precipitation and sequenced using the BigDye version 3.1 kit (Applied Biosystems, Foster City, California) on an ABI 3130 sequence analyzer (Applied Biosystems). Both strands of the amplified DNA fragments were sequenced, edited in Sequencher version 4.10.1 (Gene Codes, Ann Arbor, Michigan), and manually corrected for ambiguous base calls.

Sequence analysis: DNA sequences were aligned by eye, using Sequencher, to other sequences of species of *Paragordius* and *Chordodes* in the GenBank database or our unpublished sequences. CO1 genetic distances between each pair of samples were calculated using the Kimura 2-parameter (K2P) model (Kimura 1980) in MEGA version 5 (Tamura *et al*. 2011). All sequences from this study have been deposited in GenBank (accession numbers KU721072-KU721073).

Infected snail distribution model development: Variables assumed to be important for freshwater snail distribution were chosen as environmental input data for ecological niche modeling and included (1) elevation, (2) stream flow direction, (3) stream flow accumulation, (4) land use data, and (5) distance from stream. Environmental variables for elevation and land use land cover were downloaded for the state of Oklahoma from the United States Department of Agriculture Natural Resources Conservation Service website (http://datagateway.nrcs.usda.gov/GDGOrder.aspx) as 90 m resolution GIS layers. We then used the hydrology tool in ArcGIS 10 (ERSI, Redlands, CA) to generate stream flow accumulation data and stream flow direction data from the downloaded elevation layer. Finally, distance from stream was generated in ArcGIS 10 within a 500 m radius around each stream. All five environmental layers were then clipped to the extent of Payne County.

Niche models were constructed using the maximum entropy method implemented in Maxent 3.3.0 beta (http://www.cs.princeton.edu/~schapire/MAXENT/). A model was constructed to predict the distribution of snails with gordiid infection using 32 occurrence points (positive sites for gordiid cysts/larvae in snails). Additionally, a model was generated using locations with snails uninfected with gordiids using 14 occurrences (negative sites for gordiid cysts/larvae). This model was then used as a mask to further refine the original gordiid infection model by accounting for uninfected snail hosts that would confound the prediction for gordiid distribution. Default Maxent parameter settings were used according to Phillips *et al.* (2006) and Phillips & Dudik (2008). The available occurrence data was split to train and test models generated: 50 % of the infected snail presence data was used to train and 50% to test the gordiid model; whereas 70 % of the uninfected snail presence data was used to train and 30% to test the uninfected snail model. For all models, the accuracy of the niche model was estimated using the receiver operating characteristic (ROC) and associated area under the ROC curve (AUC) statistic, calculated in Maxent (Peterson *et al.* 2008). Briefly, the ROC analysis involves plotting the true positive rate (specificity; presence records predicted present) against the false positive rate (1-specificity; absence records predicted present). The AUC statistic is then compared against the null expectation of 0.5 (Peterson *et al.* 2008). Finally, the omission rate corresponding to the minimum training omission threshold was calculated by Maxent and was used to assess how many test occurrence points were predicted absent by the model (Peterson *et al*. 2008).

The minimum training omission threshold, the Maxent suitability value at which all training occurrences are predicted present, was used to reclassify Maxent continuous output files to binary format with 0 (absence) and 1 (presence) values, for both the gordiid model and the uninfected snail model. To generate a layer which would mask out the areas suitable for uninfected snails, we used the uninfected snail map reclassified to 1 (presence of uninfected snails) and NoData (absence of uninfected snails). Next, we used the Extract by Mask tool in ArcGIS 10 to apply the mask on the map of the gordiid model. The Extract Values to Points tool was used to confirm if occurrence points were predicted as present. A 250 m buffer around streams was applied to the model for clearer visual representation. Finally, all occurrence points where adult free-living worms were collected were plotted onto the map of the ecological niche model.

Results

Cyst/larvae morphology and distribution: A total of 1,015 snails was collected from 46 sites (Table 1). Of those, 197 (19.4 %) were infected with a total of 1,749 cysts and 48 unencysted larvae (MI = 9.08; range = 1–262). The average snail length and width was 8.4 ± 2.3 mm (2–17) and 4.8 ± 1.3 mm (1–10), respectively. There was no significant correlation between snail length or width and total cyst/larva gordiid intensity or abundance at any of the individual sites or the overall collection of snails (all $P > 0.05$). The smallest infected snail was 5 mm in length and 3 mm in width and contained 32 cysts; whereas the largest infected snail was 17 mm in length by 9 mm in width and contained one cyst. Of the 46 localities sampled, 32 (70 %) contained at least one infected snail with at least one gordiid cyst and/or larva (Table 1). Three types of gordiid cysts and three types of gordiid larvae were identified, and these included *Paragordius* spp., *Gordius* spp. and *Chordodes*/*Neochordodes* spp. (Fig. 1). Prevalence, mean intensity, and mean abundance for overall snail infections with gordiids and identified gordiid cysts/larvae type at each location are reported in Table 1. Cysts and larvae of *Paragordius* spp. were the most commonly encountered, occurring at 50 % (23/46) of the sites (Fig. 2A) and accounted for 97 % (1,702/1,749) of the cysts and 58 % (28/48) of the larvae recovered. Cysts and larvae of *Gordius* spp. were the second most commonly encountered gordiids, and these were found at 26 % (12/46) of the sites (Fig. 2B) and accounted for 1.7 % (31/1,749) of the cysts and 4 % (2/48) of the larvae recovered. Finally, cysts of *Chordodes/Neochordodes* spp. were the least encountered gordiids in this study and were found at 11 % (5/46) of the sites (Fig. 2C) and accounted for 0.2 % (4/1,702) of the cysts and 16 % (8/48) of the larvae recovered. Twelve cysts (0.7 %) and 10 larvae (21 %) could not be identified to type do to their position in snail tissue or because they were obscured by snail tissue. When considering only identified gordiid cysts/larvae, richness of gordiid cyst/larval type per individual snail was 0.2 ± 0.41 (0-2) and seven of 46 localities (15 %) contained multiple gordiid cyst/larval types. Four sites were positive for *Paragordius* spp. and *Gordius* spp. cysts and/or larvae, two sites were positive for *Paragordius* spp. and *Chordodes*/*Neochordodes* spp. type gordiid cysts and/or larvae, and one site was positive for *Gordius* spp. and *Chordodes*/*Neochordodes* spp. type gordiid cysts and/or larvae (see Table 1). However, only 2 % of individual snails (4/197) were infected with multiple gordiid cysts and/or larval types. One snail from site 9 was infected with four *Paragordius* spp. cysts and two *Gordius* spp. cysts, and one snail from site 24 was infected with two *Paragordius* spp. cysts and one *Gordius* sp. cysts. Finally, two snails from site 18 had multiple gordiid cyst/larvae infections and these included one snail with six *Paragordius* spp. larvae/cysts and two *Chordodes* spp. larvae; whereas the other snail was infected with one larva each of *Paragordius* sp. and *Chordodes* sp.

Cricket infections, adult free-living worm morphology, and sequence analyses: Sixty of the 80 *A. domesticus* crickets exposed to *Paragordius* spp. cysts from the four sites survived long enough to release adult worms (Fig. 3). Crickets released a total of 144 (93 female and 51 male) worms 34–38 days post exposure. These included 26 females and 15 males from site 24; 39 females and 18 males from site 27; 22 females and 12 males from site 33, and six females and six males from site 40. Based on morphology, all adult worms were identified as *P. varius*. Briefly, both male and female worms contained a white calotte followed by a dark collar on the anterior end. Female worms were yellowish brown in color and 140-160 mm in length and 600-850 µm in width. The female posterior end was divided into three tail lobes, including one dorsal (0.83 mm in length by 0.25 mm in width) and two lateroventral lobes (0.84 mm in length by 0.28 mm in width; Fig. 4A). Male worms were yellowish brown to almost black in color, and 110-122 mm long by 400-700 µm wide. The posterior ends of males contained two long and slender tail lobes (0.4 mm in length by 0.15 mm in width) and the ventral midline anterior to the

cloacal opening was bordered on both sides by long rows of short spines (Fig. 4B). The cuticle of male and female worms was structured by transverse irregular cords separated by furrows of varying depths. Cords were elevated and contained hemispherical structures on top (Fig. 4c). The CO1 sequence, containing 658 bp, matched most closely that of other *P. varius* individuals collected in Nebraska and Mississippi. Genetic distance between the three individuals was 0.20 %.

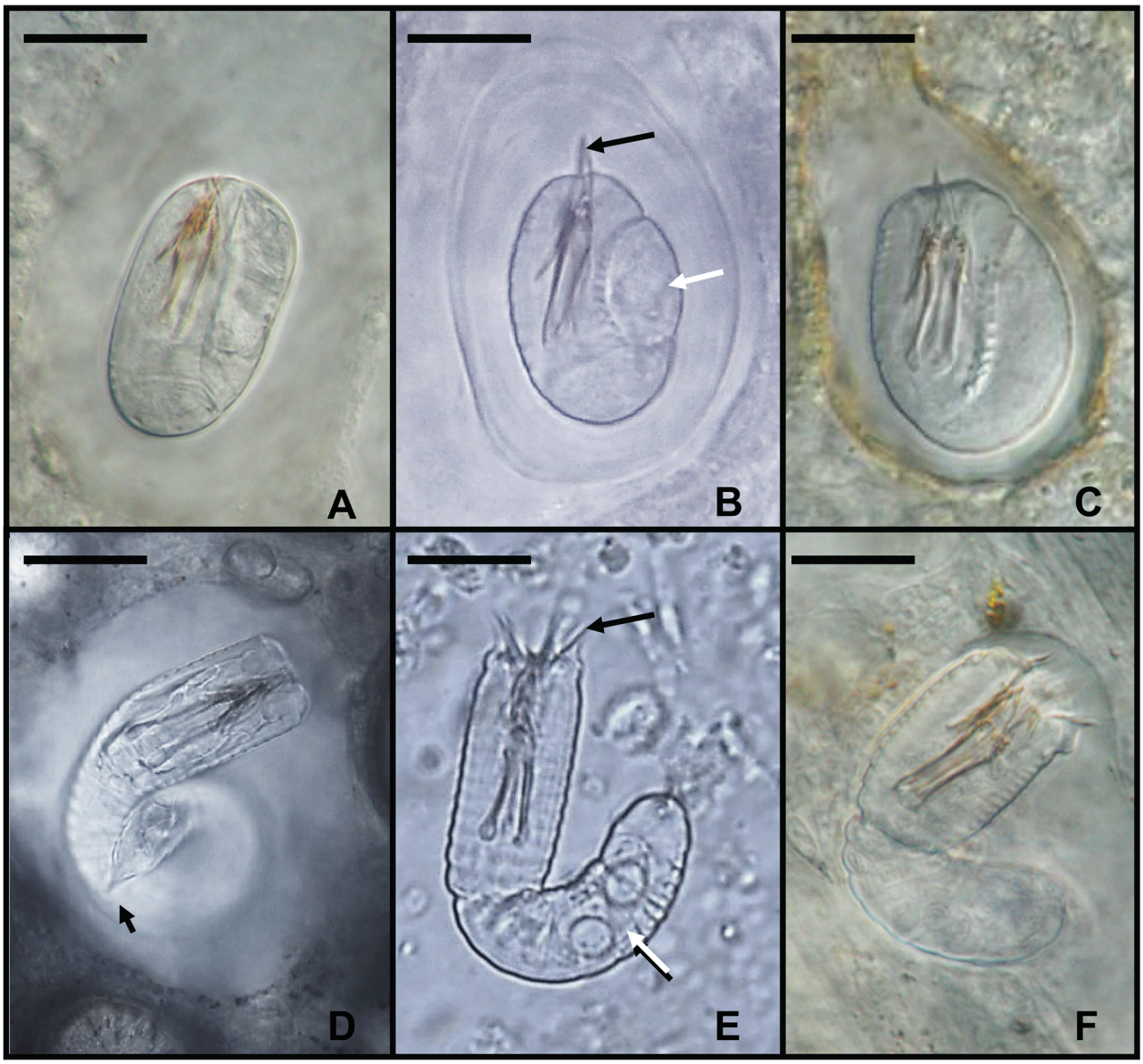

FIGURE 1. Photomicrographs of gordiid cyst and larval types recovered from *Physa acuta* snails collected in Payne County, Oklahoma. (**A**) Cyst of *Gordius* sp. Note the lack of spines on the preseptum and tighly double folded larva. Scale bar = 25 µm. (**B**) Cyst of *Paragordius* sp. Note the characterisitc spines on the preseptum (black arrow) and double folding of the postseptum (white arrow) never reaching the posterior end of the preseptum. Scale bar = 15 µm. (**C**) Cyst of *Chordodes*/*Neochordodes* sp. Note the single folding position of the postseptum and relatively small spines on the preseptum. Scale bar = 10 µm. (**D**) Larva of *Gordius* sp. in the process of folding. Note the characteristic single spine on the posterior end of the postseptum (arrow). Scale bar = 15 µm. (**E**) Unencysted larva of *Paragordius* sp. Note the characteristically long spines of the outer hooks of the preseptum (black arrow) and characteristic granules of the pseudointestine (white arrow). Scale bar = 15 µm. (**F**) Unencysted larva of *Chordodes*/*Neochordodes* sp. Note the equal length ratio of the prespeptum and postseptum and relatively small spines on the preseptum. Scale bar = 10 µm.

TABLE 1. Site, location, prevalence, mean intensity (range), mean abundance ± 1SD, and type of gordiid cysts/larvae infecting *Physa acuta* from 46 locations in Payne County, Oklahoma.

Site	Latitude	Longitude	Prevalence % (no. infected/no. examined)	MI (Range)	MA ± SD	Type of gordiid cyst/ larva found
1	36.144817	-97.165133	0 (0/22)	–	0	–
2	36.130307	-97.103840	0 (0/19)	–	0	–
3	36.130094	-97.140577	0 (0/20)	–	0	–
4	36.130822	-97.056425	0 (0/20)	–	0	–
5	36.174072	-97.027967	10 (2/19)	1 (1)	0.1 ± 0.3	G
6	36.174048	-97.029150	9 (2/21)	1 (1)	0.1 ± 0.3	G
7	36.154984	-97.033909	4 (1/23)	1 (1)	0.05 ± 0.2	C
8*	36.115864	-97.007432	16 (4/24)	1.3 (1–2)	0.2 ± 0.5	C, G
9*	36.115933	-97.013226	12 (3/25)	3 (1–6)	0.4 ± 1.3	G, P
10	36.202330	-96.997776	10 (2/20)	1 (1)	0.1 ± 0.3	G
11	36.189875	-96.980122	4 (1/22)	1 (1)	0.04 ± 0.2	G
12	36.218014	-96.981210	0 (0/17)	–	0	–
13	36.217099	-97.135255	0 (0/20)	–	0	–
14	36.246350	-96.975889	15 (3/20)	2 (1–4)	0.3 ± 0.9	C
15	36.195110	-96.961899	10 (2/20)	1 (1)	0.1 ± 0.3	G
16	36.043487	-97.122911	0 (0/20)	–	0	–
17	36.043476	-97.107790	0 (0/20)	–	0	–
18*	36.028781	-97.155962	60 (14/23)	2.5 (1–10)	1.6 ± 2.3	C, P
19	36.109242	-97.256255	5 (1/20)	1 (1)	0.05 ± 0.2	C
20	36.100400	-97.228639	0 (0/20)	–	0	–
21	36.028833	-97.193427	5 (1/20)	12 (12)	0.6 ± 2.6	P
22	36.116106	-96.867571	25 (5/20)	1 (1)	0.3 ± 0.4	P
23	36.113125	-96.743953	34 (8/23)	2.1 (1–3)	0.7 ± 1.2	P
24*	36.116671	-96.647716	85 (17/20)	17.8 (2–74)	15.2 ± 17.8	G, P
25	36.016800	-96.714138	4.7 (1/21)	1 (1)	0.05 ± 0.3	G
26	36.000200	-96.754616	30 (6/20)	1.8 (1–4)	0.6 ± 1.1	G
27*	36.043842	-96.846392	80 (16/20)	24.6 (1–262)	19.7 ± 58.9	C, P
28	36.044219	-96.85695	10 (2/20)	1 (1)	0.1 ± 0.3	P
29	35.983644	-96.933778	5 (1/19)	1 (1)	0.1 ± 0.2	P
30	35.995583	-96.998450	31 (6/19)	1 (1)	0.3 ± 0.5	P
31	36.057860	-97.316787	0 (0/20)	–	0	–
32	36.017362	-97.318332	22 (5/22)	13 (3–-44)	3.0 ± 9.5	P
33	36.102088	-96.939398	100 (20/20)	14.5 (1–61)	14.5 ± 14.5	P
34	36.026518	-96.696685	28 (6/21)	3.2 (1–10)	0.9 ± 2.4	P
35*	35.982295	-96.696532	15 (3/20)	1.3 (1–2)	0.2 ± 0.5	G, P
36	35.970973	-96.741571	0 (0/20)	–	0	–
37	35.955829	-96.906581	83 (15/18)	4.7 (1–25)	3.9 ± 6.2	P
38*	36.014000	-96.944965	10 (2/20)	1 (1)	0.1 ± .3	G, P
39	36.107950	-97.264833	0 (0/20)	–	0	–
40	36.014503	-97.245800	100 (20/20)	20.9 (1–145)	20.9 ± 33.5	P
41	35.970921	-97.155600	70 (14/20)	5.7 (1–18)	4 ± 4.8	P

.....continued on the next page

TABLE 1. (Continued)

Site	Latitude	Longitude	Prevalence % (no. infected/no. examined)	MI (Range)	MA ± SD	Type of gordiid cyst/ larva found
42	35.999917	-97.124226	35 (7/20)	1.6 (1–3)	0.6 ± 0.9	P
43	36.189222	-96.953273	0 (0/20)	–	0	–
44	36.094644	-96.872463	0 (0/20)	–	0	–
45	36.055951	-96.873343	15 (3/20)	1.3 (1–2)	0.2 ± 0.5	P
46	36.043564	-96.981940	20 (4/20)	3.8 (1–8)	0.8 ± 2.0	P

* = sites with multiple gordiid cyst types, C = *Chordodes*/*Neochordodes* spp., G = *Gordius* spp., and P = *Paragordius* spp.

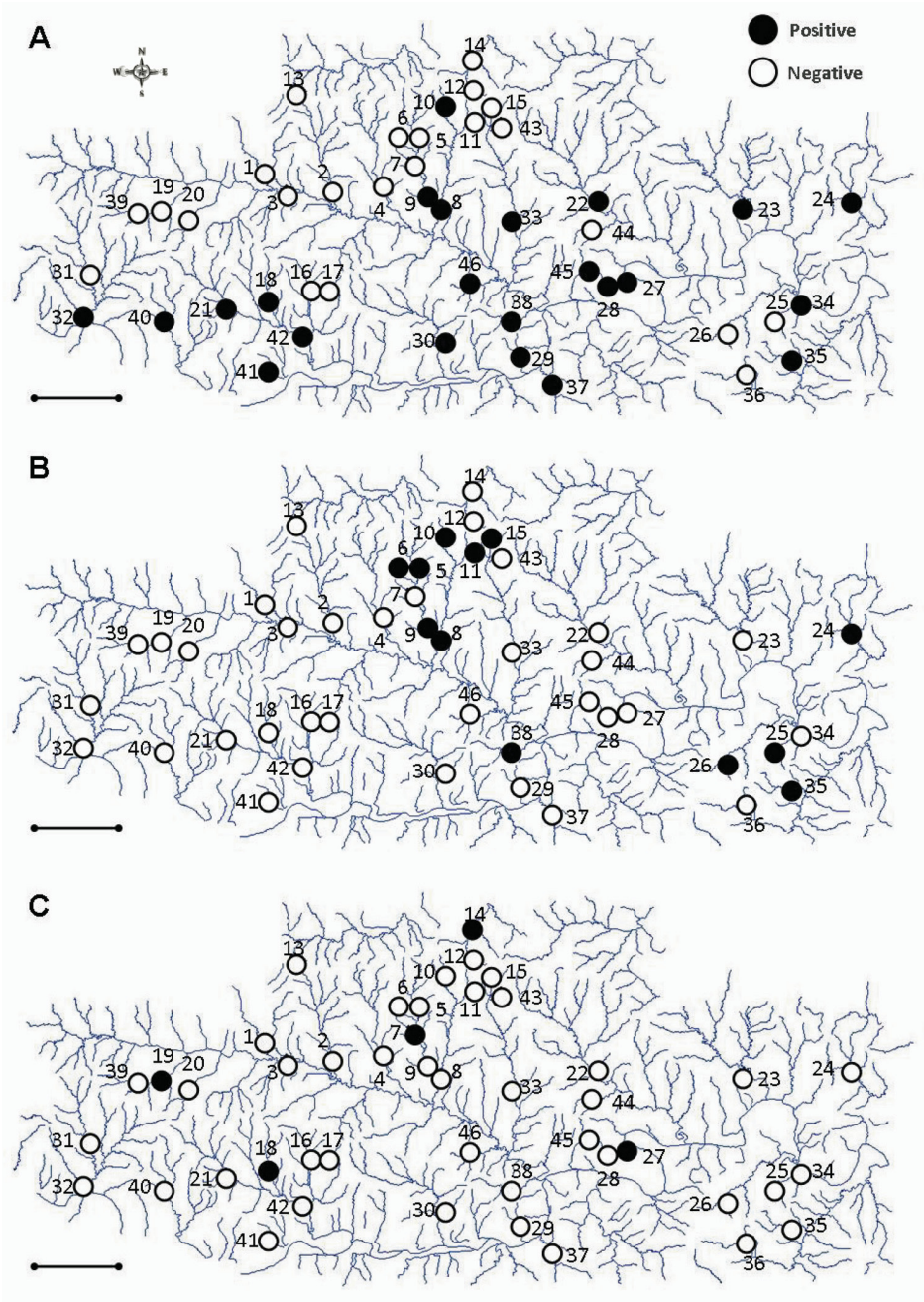

FIGURE 2. Positive (black circles) and negative (white circles) localities for (**A**) *Paragordius* spp., (**B**) *Gordius* spp., and (**C**) *Chordodes*/*Neochordodes* spp. in Payne County, Oklahoma. Numbers represent names of each site (see Table 1). Scale bars = 6.9 km.

FIGURE 3. (**A**) Female *Acheta domesticus* releasing a single female *Paragordius varius*. (**B**) A gordian knot of male and female *P. varius* after emerging from a female *A. domesticus*. Note that males are the darker and thiner individuals. Scale bars = 1 cm.

Additionally, adult free-living worms of two other gordiid species were collected by us or citizen scientists from five locations in and surrounding Stillwater, Oklahoma. These included 38 males and three females of *Gordius* **n. sp.** collected from wet lawns during December 2011-February 2012 and a single male *C. morgani* collected from a dog water dish in May of 2011 (Table 2).

TABLE 2. Site, date collected, location, species, and number of free-living male and female gordiids collected from 5 locations in Payne County, Oklahoma.

Site	Date	Latitude	Longitude	Species	No. males	No. females
A	May 6, 2011	36.126126	-96.918667	*Chordodes morgani*	1	0
B	December 15, 2011	36.127332	-97.033850	*Gordius* **n. sp.**	13	0
C	January 14, 2012	36.121435	-97.126883	*Gordius* **n. sp.**	18	1
D	February 1, 2012	36.101417	-97.088659	*Gordius* **n. sp.**	6	2
E	February 3, 2012	36.132569	-96.989869	*Gordius* **n. sp.**	1	0

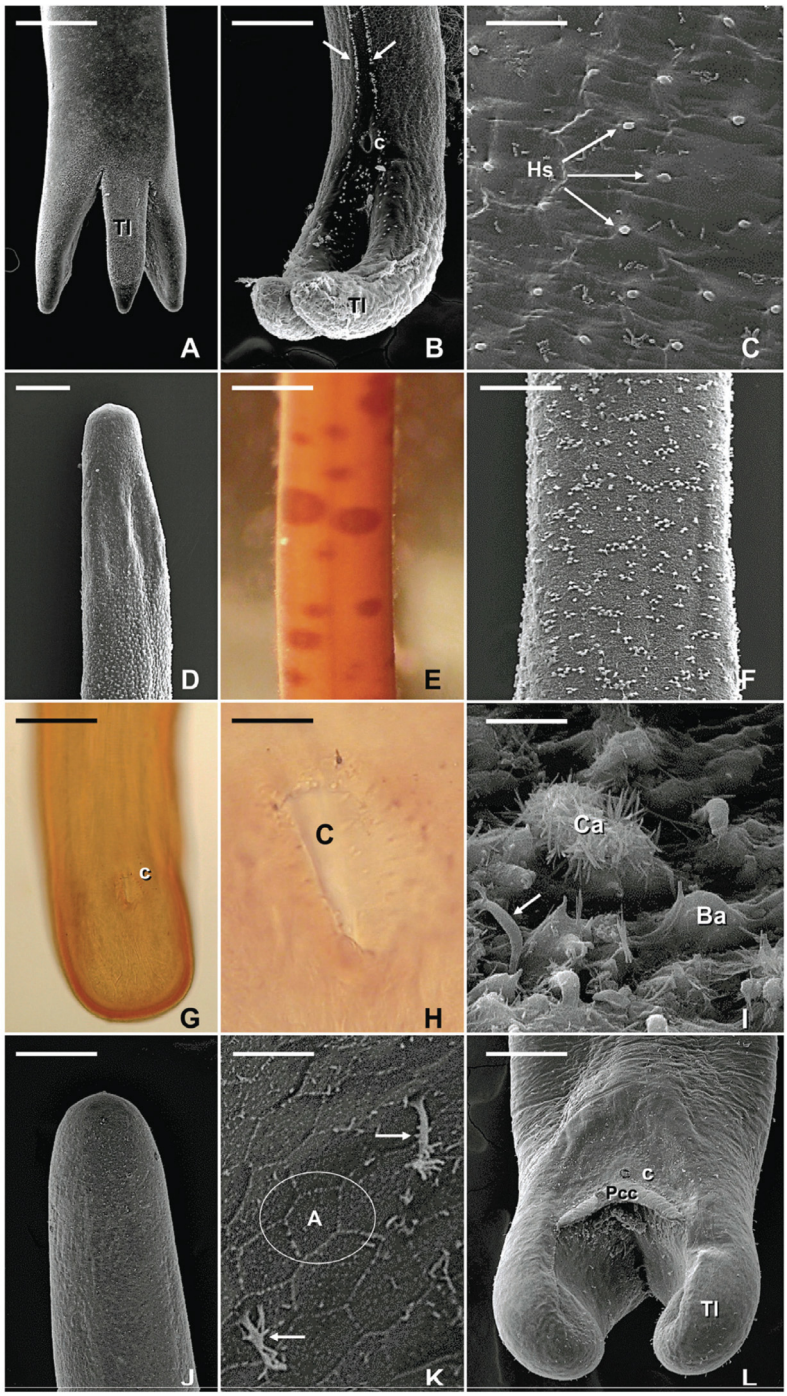

FIGURE 4. Scanning electron and light microscope micrographs of adult free-living gordiids reared from field collected cysts or collected as free-living adults from Payne County, Oklahoma. (**A**) Dorsal view of the posterior region of a female *Paragordius varius*. Note the three posterior tail lobes (Tl). Scale bar = 500 µm. (**B**) Ventral view of the posterior region of a male *Paragordius varius*. Note the two long posterior tail lobes (Tl), cloaca (c), and spines on the ventral midline (white arrows). Scale bar = 200 µm. (**C**) Midbody cuticular structures of a male *P. varius*. Note the hemisphirical structures (Hs). Scale bar = 10 µm. (**D**) Tapered anterior end and cuticular patern of a male *Chordodes morgani*. Scale bar = 100 µm. (**E**) Midbody region of a male *C. morgani* showing the characteristic leopard pattern. Scale bar = 300 µm. (**F**) Midbody region of a male *C. morgani* showing the characteristic crown areole pattern. Scale bar = 100 µm. (**G**) Ventral view of the posterior region of a male *C. morgaini*. Note no tail lobes and an oval cloaca (c). Scale bar = 200 µm. (**H**) Cloaca opening surrounded by circumcloacal spines. Scale bar = 10 µm. (**I**) Cuticular structures on the posterior region of a male *C. morgaini*. Note the crown areoles (Ca), bulging areoles (BA), and bristles (white arrow). Scale bar = 8 µm. (**J**) Tapered anterior end and cuticular patern of a male *Gordius* **n. sp.** Scale bar = 10 µm. (**K**) Areole pattern on the posterior body region of a male *Gordius* **n. sp.** Note the weakly developed areoles (A) and the presence of bristles (white arrows). Scale bar = 20 µm. (**L**) Ventral view of the posterior region of a male *Gordius* **n. sp.** Note the two posterior tail lobes (Tl), cloaca (c), and post cloacal crescent (Pcc). Scale bar = 120 µm.

The single male *C. morgani* was 180 mm in length and 400 µm in width. The anterior end was tapered (Fig. 4D) and the anterior tip was white in color. The cuticle was light brown in color with dark brown patches characteristic of the leopard pattern for the genus (Fig. 4E) and the cuticle contained patches of elevated and crown areoles (Fig. 4F). The posterior end was not divided into lobes (Fig 4G), but a ventral furrow indicated bilobation. The cloacal opening was oval and contained circumcloacal spines (Figs. 4G and H). Bristles, bulging areoles, and crowned areoles were numerous and extended along the lateral sides of the posterior end (Fig. 4I). The CO1 sequence, containing 658 bp, matched most closely with *C. morgani*. The sequence was compared to others from individuals of *C. morgani* collected in Arizona, Florida, New Mexico, and Nebraska. Genetic distance between all eight *C. morgani* individuals was 2.23 %.

Male and female free-living *Gordius* **n. sp.** were yellowish to light brown in color, and 164–223 mm long by 600–700 µm wide and 155–333 mm long by 780–900 µm wide, respectively. The anterior end was tapered (Fig. 4J) and contained a white calotte followed by a dark collar. The cuticle was variable among individuals but in most individuals it contained one type of areole with various bristles distributed on the anterior, midbody, and/or posterior regions of the worms (Fig. 4K). Areoles were weakly developed, polygonal in shape, and 8–12 µm in diameter (Fig. 4K). The posterior end of males contained two terminal tail lobes (0.2 mm in length by 0.12 mm in width) and a postcloacal crescent was present (Fig. 4L). The circular cloacal opening in males was round and situated in a broad nonareolar field (Fig. 4L). Females possessed a round posterior end, cylindrical in shape, with a terminal cloaca (not shown).

Diagnosis and comments: All individuals of *P. varius* and *C. morgani* from Oklahoma examined with a SEM or LM conformed to previous descriptions of these species (Schmidt-Rhaesa *et al.* 2003). Additionally, our molecular analyses indicated that *P. varius* collected from three distinct locations only varied in its CO1 genetic distance by 0.20 %; whereas the genetic distance between eight individuals of *C. morgani* collected from five locations varied by 2.23 %. Although little is known about the genetic markers in this group of parasites, previous work on the genus *Gordius* (Hanelt *et al*. 2015) indicates that this level of CO1 intraspecific variation (below 3 %) is consistent with findings in other hairworm species and similar to the intraspecific genetic variation in nematodes, the sister phylum to the Nematomorpha. For example, in free-living freshwater and marine nematodes, intraspecific distances are below 2.5 % and 5 %, respectively; whereas interspecific genetic distances are above 7 % and 5 %, respectively (Derycke *et al*. 2010, Ristau *et al*. 2013). Additionally, in parasitic filarioid nematodes, interspecific distances are above 7 %, averaging about 15%, and intraspecific distances are below 2.5% (Ferri *et al*. 2009).

In contrast to *P. varius* and *C. morgani*, all individuals of *Gordius* **n. sp.** collected in this study from Oklahoma and identified using LM superficially resembled the most commonly reported North American gordiid, *G. robustus*. However, based on areole morphology using SEM, the Oklahoma *Gordius* **n. sp.** differed morphologically from the description of *G. robustus*, which has a smooth cuticle, and from descriptions of *Gordius difficilis* Montgomery, 1898 and *Gordius attoni* Redlich, 1980, both of which have polygonal areoles that are distinct from the Oklahoma *Gordius* **n. sp.** collected during this study (Bolek & Coggins 2002; Schmidt-Rhaesa *et al.* 2003). In addition, published molecular data on *G. robustus* collected from 28 localities across North America, including individuals from Oklahoma, indicate that *Gordius* cf. *robustus* is comprised of at least eight different species (Hanelt *et al.* 2015). From our previous work, mitochondria CO1 genetic distances indicate that *Gordius* **n. sp.** from Oklahoma differs by 8–21 % in the CO1 genetic distance from the other seven *G.* cf. *robustus* and only 1.5 % within individuals collected from Oklahoma, Texas, and Louisiana (Hanelt *et al.* 2015). However, currently little information is available on the morphology of the other seven species in the *G.* cf. *robustus* complex. As a result, we will formally describe *Gordius* **n. sp.** in a future publication when morphological data are available for the other seven species in the *G.* cf. *robustus* complex.

Models for presence of gordiids in Payne County, Oklahoma: The model for uninfected snails had a higher AUC statistic score than the model for gordiid occurrences (0.915 *vs* 0.821). However, the omission rates were 0 for both models. Visually, the models appeared antagonistic, and areas predicted strongly suitable for gordiids had a low probability of occurrence of uninfected snails. After applying the mask of uninfected snails to the gordiid prediction map, the gordiids distribution was further refined. Environmentally suitable areas were located in the southwest and central portion of Payne County, whereas areas in the northwestern and west-central portion of the county were environmentally unsuitable for snail infections with gordiids (Fig. 5). Mapping the five localities from where free-living worms were collected on to the model indicated that four of the five locations were found in areas

predicted environmentally suitable for gordiids by the model. Only a male *C. morgani* was not collected from an area predicted suitable for gordiids. However, this individual was collected from an unnatural environment (dog water bowl) from a house located in the vicinity of an environmentally suitable area predicted by the model (Fig. 5).

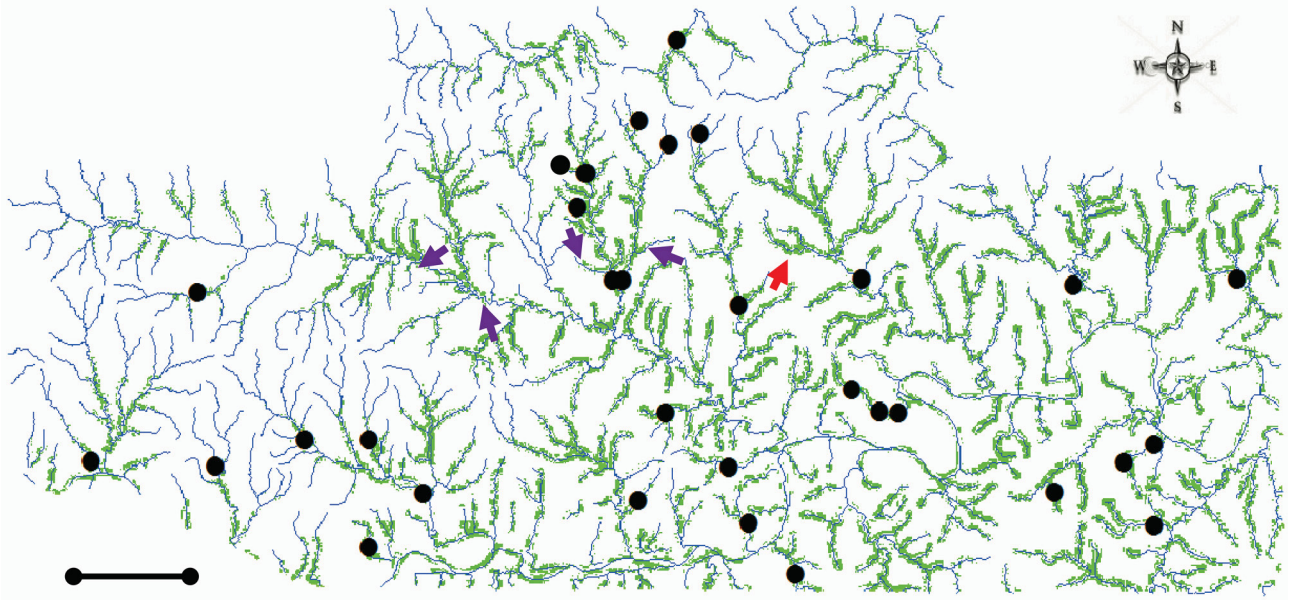

FIGURE 5. Maxent model summary of gordiid infected snail geography showing known occurrence points (black circles) and predicted potential geographic distribution (green areas of streams) in Payne County, Oklahoma. Blue areas of streams are identified as unsuitable. Purple and red arrows indicate locations of free-living adults of *Gordius* **n. sp.** and *Chordodes morgani*, respectively. Note that all *Gordius* **n. sp.** were collected in the predicted geographic distribution, and *C. morgani* was collected from a dog water bowl from a home near a region of a stream with predicted distribution for gordiids. Scale bar = 6.7 km.

Discussion

Progress in our understanding of the diversity and distribution of hairworms has been hindered by the difficulty of locating and collecting adult free-living worms. However, our work along with the work of Hanelt *et al.* (2001) on the distribution of non-adult stages of gordiids is overcoming these difficulties. In this study, we were able to successfully show that sampling for gordiids by using cyst and/or larval stages in snail hosts is an effective tool for rapid assessment of gordiid distribution over a large geographic area. By sampling a single county in Oklahoma we were able to show that gordiids were very common and widely distributed in the environment. In fact, 70 % of sites sampled in our study area were identified as positive for the presence of gordiids. As in our study, Hanelt *et al.* (2001), in a three-year study of 50 streams in a 2,000 km^2 area of the Western Corn Belt Plains Ecoregion of Nebraska, found that 70 % of their sampled sites were positive for the presence of gordiids. This is particularly important because Hanelt *et al.* (2001) also sampled for adult worms at each of their locations and only found three adult worms at a single site. In our study, and during our snail surveys, we did not find any adult free-living worms at any of our locations, even though multiple searches were conducted for adult free-living worms at some of our sites (data not shown). Taken together, these data clearly indicate that, compared to free-living adult worms, the cyst stages of gordiids in aquatic snails are the most common stages of gordiids to detect in the environment.

More importantly, our study presents an additional technique for discovering the hidden diversity of gordiids. Because Hanelt *et al.* (2001) conducted their study when little was known of the morphological characteristics of non-adult stages of gordiids (larvae and cysts), and they were unable to identify gordiid cysts in the snails sampled. However, using morphological characteristics of gordiid cyst and/or larvae, we were able to differentiate among gordiid genera and/or clades and in the process increased our resolution of the potential biodiversity of hairworms in a particular geographic area. Using morphological characteristics of cyst and larvae we were able to differentiate among three genera/clades of gordiids, including *Gordius* spp., *Paragordius* spp., and *Chordodes/Neochordodes* spp.

Of the three genera/clades of gordiids identified by cysts and/or larvae in this study, the genus *Paragordius* was the most commonly encountered and was the most abundant non-adult stage of gordiids found in this study. Currently, only a single species of *Paragordius* (*P. varius*) and a single species of *Chordodes* (*C. morgani*) are known from the United States (Schmidt-Rhaesa *et al*. 2003), and our cyst data, together with our morphological and molecular data for adult free-living worms, indicate that *P. varius* was one of the most commonly distributed gordiids; whereas *C. morgani* was more restricted in its distribution in Payne County, Oklahoma. Cysts and/or larvae of *Paragordius* occurred in the central and southern regions of Payne County, cysts and/or larvae of *Gordius* were distributed in the northern and eastern regions, whereas cysts of *Chordodes/Neochordodes* occurred in the north-central and western regions of Payne County. One explanation for these observed differences in occurrence data among the cysts and/or larvae of these gordiid genera/clades may be related to the distribution and abundance of their definitive arthropod hosts.

Unfortunately, little information is available on the definitive arthropod host use and specificity for most gordiid species. In fact, of the 350 described gordiid species, arthropod hosts are known for less than 100 (29%) species of gordiids, and currently the definitive arthropod host for *Gordius* **n. sp.** is unknown (Schmidt-Rhaesa 2013). However, other North American species of *Gordius* have been reported from ground beetles (Carabidae), three families of orthopterans (Tettigonidae, Stenopelmatidae, and Acrididae) and the millipede *Narceus americanus* (Palisot de Beauvois 1817) (Schmidt-Rhaesa *et al*. 2003; Poinar & Weismann 2004; Schmidt-Rhaesa 2013; Hanelt *et al.* 2015). More importantly, our work on the biogeography of the *G.* cf. *robustus* complex strongly suggests that most species of North American *Gordius* are restricted to one or a few closely related arthropod hosts (Hanelt *et al.* 2015). In contrast, *C. morgani* has been reported from cave and camel crickets (Rhaphidophoridae) and three species of cockroaches from two families (Blattidae and Ectobidae); whereas all members of the genus *Paragordius*, for which definitive arthropod hosts are known, infect orthopterans in the family Gryllidae, and all confirmed reports indicate that *P. varius* infects crickets in the genera *Acheta* Linné, 1758, *Gryllus* Linné, 1758, and *Allonemobius* (De Geer, 1773) throughout North America (Schmidt-Rhaesa *et al.* 2003; Hanelt & Janovy 2004; Poinar & Weismann 2004; Hanelt *et al*. 2012; McAllister & Hanelt 2012). Because currently no information is available on the distribution and abundances of these arthropod hosts in Payne County, Oklahoma, it is difficult to make any predictions about the observed differences in occurrence data among the three groups of gordiids found in this study.

Although gordiid host specificity and distribution can vary among species, what is consistent among all hairworm species is the requirement for an aquatic habitat for reproduction. Once worms emerge in an aquatic habitat from their definitive arthropod hosts, they mate and females deposit egg strings (Schmidt-Rhaesa 1997; Bolek *et al*. 2015). Once hatched, larvae reside in the sediment where they are consumed and encyst in various aquatic animals. Unpublished data from our laboratory indicate that *P. acuta* snails are equally susceptible to infections by all species of gordiids in the genera *Gordius*, *Paragordius*, *Chordodes*, and *Neochordodes*. Although aquatic snails are usually not ingested by terrestrial arthropods, which serve as definitive hosts for gordiids, these observations suggest that the presence of infected snails with gordiid cysts and/or larvae can be useful for predicting the completion of gordiid life cycles in the environment. By using occurrence points for infected snails we were able to estimate the potential distribution of gordiids in infected snails in Payne County, Oklahoma. Importantly, these predictions were able to recover all known occurrence points. Areas that were highly suitable were located primarily in the watershed of the central, southern, and eastern parts of the county; whereas areas in the western part of the county were not predicted to be suitable for snail infections with gordiids, indicating that gordiids are not equally distributed in the environment. More importantly, four of the five locations where adult free-living worms were collected were predicted suitable by our model. Although *C. morgani* was not collected from a predicted area for gordiids, this individual was collected from an unnatural environment (dog water bowl) from a house located in the vicinity of an area predicted environmentally suitable by the model. Taken together, these observations indicate that using cyst occurrence data to develop ecological niche models can be an additional tool for predicting environmentally suitable areas for the presence of free-living adult gordiids and discovering new species of these cryptic parasites.

Our work indicates that sampling for non-adult stages of gordiids can greatly increase our knowledge on the hidden diversity of these parasites. In the future, as more information is acquired on non-adult stages of other genera and species of gordiids and more environmental layers become available at a finer scale, these tools can be used in conjunction to help us discover some of the biological reasons for the distribution of gordiids in the environment.

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